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Proteome analysis of the m. *longissimus dorsi* between fattening stages in Hanwoo steer

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The objective of this study was to identify proteins in the m. *longissimus dorsi* between early (12 months of age) and late (27 months of age) fattening stages of Hanwoo (Korean cattle) steers. Using two-dimensional electrophoresis and mass spectrometry, 8 proteins of 11 differentially expressed spots between the 12 and 27 month age groups were identified in the loin muscle. Among those that were differentially expressed, zinc finger 323 and myosin light chain were highly expressed in late-fattening stage, and two catabolic enzymes, triosephosphate isomerase (TPI) and succinate dehydrogenase (SDH) were expressed more in the early versus the late-fattening stage. In particular, the quantification of TPI and SDH by immunoblotting correlated well with fat content. Our data suggested that TPI and SDH are potential candidates as markers and their identification provides new insight into the molecular mechanisms and pathways associated with intramuscular fat contents of bovine skeletal muscle. [BMB reports 2009; 42(7): 433-438]

INTRODUCTION

Marbling (intramuscular fat) is a major indicator of the juiciness and flavor of beef as well as a main profitability factor for producers in the Korean beef industry. Intramuscular fat is a main contributor to meat quality and is affected by many factors including breed, genotype, gender, age, and nutrition (1, 2). The development of intramuscular fat has been associated with fatty acid oxidation and ATP synthesis that occurs in muscle tissue (3). Previous studies have suggested that the intramuscular fat content in rabbits and sheep was due to a balance between catabolism and anabolism rather than a specific biochemical pathway (4, 5). However, others have reported a significant correlation between intramuscular fat content and the mitochondrial enzymes cytochrome c oxidase and isocitrate dehydrogenase (6).

Cattle undergo a dramatic increase in intramuscular fat during a late-finishing stage that occurs between 18 and 26 months of age (7). In Waygu (Japanese Black) cattle, approximately 20% of the total intramuscular fat is added between 12 and 26 months (8). These findings indicate that describing the changes that occur in the mitochondrial proteome between 12 and 27 months may be useful for identifying a metabolic marker for the deposition of intramuscular fat.

The objective of this study is to identify proteins that are differentially expressed in the m. *longissimus dorsi* (LD) of 12- and 27-month-old Korean cattle (Hanwoo) and to investigate their potential as biomarkers for intramuscular fat content of beef.

RESULTS AND DISCUSSION

Approximately 400 spots were detected on each silver-stained gel using a computer-assisted image analysis (Fig. 1). The majority of the protein spots were between 20 and 60 kDa. 11 spots showed a differential expression pattern and a twofold change between the 12- and 27-month-old groups (Fig. 1 and Table 1). Of the 11 spots, eight showed a higher expression pattern in the 12-month-old group, while the other three were higher in the 27-month-old group (Fig. 2). Table 1 presents the annotated 8 proteins by MS analysis.

Protein spots 6107, 7201, and 5810 had 2.6-, 4.6-fold, and 2.6-fold lower expression in the 27 month group compared to the 12 month group, respectively, and were annotated to the triosephosphate isomerase (TPI) and succinate dehydrogenase (SDH) (Table 1), which are catabolic muscle proteins. The TPI enzyme reversibly converts dehydroxyacetone phosphate (DHAP) to glyceraldehydes-3-phosphate in the glycolytic pathway (9). In adipose tissue, DHAP is synthesized from glycerol by glycerol kinase and glycerol-3-phosphate dehydrogenase. Glycerol is the main precursor for synthesis of triacylglycerol in the adipocyte (10). Thus, TPI, which controls the content of glycerol, might be associated with triacylglycerol synthesis.

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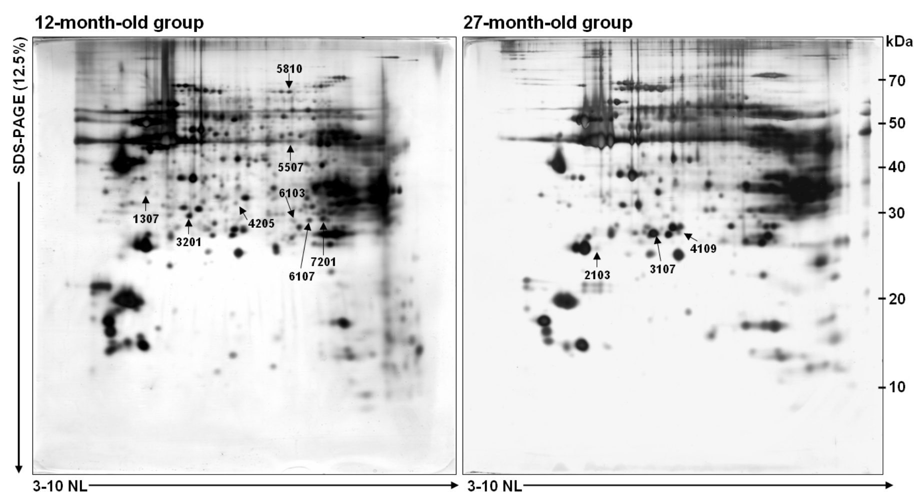

Fig. 1. Comparative analysis of expressed protein patterns between the different age group (12 and 27 months). Protein (200 μg) was loaded and separated in the IPG 3-10NL strip and a SDS gel (12.5% T). This figure shows only the parts of the 2-DE images that were useful for computer-assisted image analysis. Arrows indicate 11 spots which indicated differentially expressed proteins between the two groups. The spots were identified using the Applied Biosystems 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Table 1).

Table 1. Characterization of differentially expressed proteins between the different age groups (12 and 27 month) using the Applied Biosystems 4700 Proteomics analyzer MALDI-TOF/TOF mass spectrometer

Spot No.	Protein name	Entry name	Theoretical Mr/pI	Observed Mr/pI	Ratio[a]	Seq. Cov. (%)[b]	Method[c]
3107	Myosin light chain 1, slow-twitch muscle A isoform	MLEY_HUMAN	22.75/5.56	25.01/5.73	5.0	7	MS/MS
3201	Actin, aortic smooth muscle (Alpha-actin 2)	ACTA_BOVINE	41.98/5.23	28.32/5.48	0.4	12	MS/MS
4205	Actin, cytoplasmic 1 (Beta-actin)	ACTB_BOVINE	41.71/5.29	31.58/6.10	0.5	10	MS/MS
5810	Succinate dehydrogenase [ubiquinone] flavoprotein subunit	DHSA_BOVINE	72.89/7.55	68.64/6.78	0.3	6	MS/MS
4109	Thioredoxin-dependent peroxide reductase	PRDX3_BOVINE	28.17/7.15	25.07/6.21	2.5	10	MS/MS
6107	Triosephosphate isomerase	TPIS_RAT	26.77/6.51	26.23/7.02	0.3	16	MS/MS
7201	Triosephosphate isomerase	TPIS_MOUSE	26.56/7.08	26.23/7.21	0.2	32	MS/MS
6103	Zinc finger protein 323	ZN323_HUMAN	47.26/6.42	26.32/6.80	0.4	30	MS
1307	NI[d]	–	–	29.50/4.90	0.4	–	–
2103	NI	–	–	23.08/5.21	3.3	–	–
5507	NI	–	–	43.49/6.78	0.4	–	–

[a]Ratio value of the 27- to 12-month-old group, [b]Seq. cov., sequence coverage, [c]MS, peptide mass fingerprint; MS/MS, tandem mass spectrometry, [d]NI, not identified.

The SDH enzyme catalyzes the oxidation of succinate to fumarate as part of the Krebs cycle in mitochondria (11). Pertick *et al.* (3) reported that intramuscular fat accumulation results from a balance between fat synthesis and fat catabolism via β-oxidation, indicating that a decrease in fat catabolism is related to fat accumulation in muscle. In our study, TPI and SDH expression decreased in the 27-month-old group compared to the 12-month-old group in Hanwoo LD muscle (Figs. 1 and 2). Analysis by immunoblotting also showed 1.5 times more TPI and 2.0 times more SDH expression in the 12-month-old group (Fig. 3). Additionally, both proteins (−0.95 for TIM and −0.83 for SDH) were negatively correlated with fat content ($P < 0.01$ and 0.05) (data is not shown). These results show that proteins in a catabolic pathway were associated with more intramuscular fat in LD muscle of late fattening stage (27-month-old) steers.

The actin and myosin light chain proteins are associated with myofibrils and muscle development (12). Intramuscular fat increased during the late fattening stage (18-24 months old) (13, 14). In Hanwoo steer, the intramuscular fat content (%) was eight times higher in the 27-month-old group (1.8 ± 0.470 vs. 22.0 ± 5.240, $P < 0.01$) than in the 12-month-old group. In general, adipose tissue development was positively associated with the expression level of PPARγ genes (15). Recently, Lee *et al.* (16) suggested that PPARγ inhibited the expression of myogenic proteins and the formation of myotubes, while other studies have reported that myofibril proteins decreased during adipogenesis (17, 18). This agrees with our data indicating that myofibril protein content decreased as intramuscular fat increased during the fattening stage (Fig. 2). A

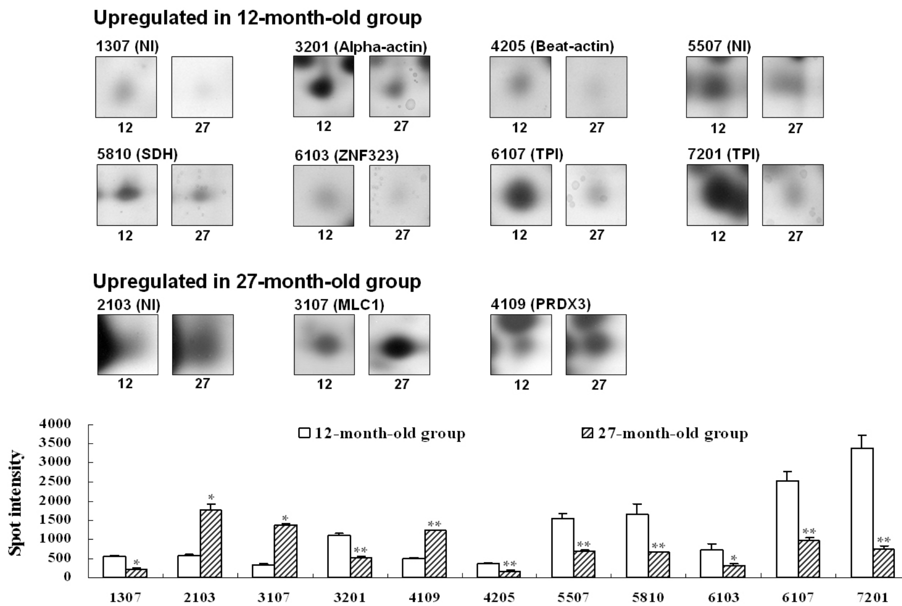

Fig. 2. Expression profiles of the differential expressed spots between the different age groups (12 and 27 months) are represented by the intensity. */**Significant differences ($P < 0.05$ and $P < 0.01$, respectively) between groups were determined using the t-test module on SAS.

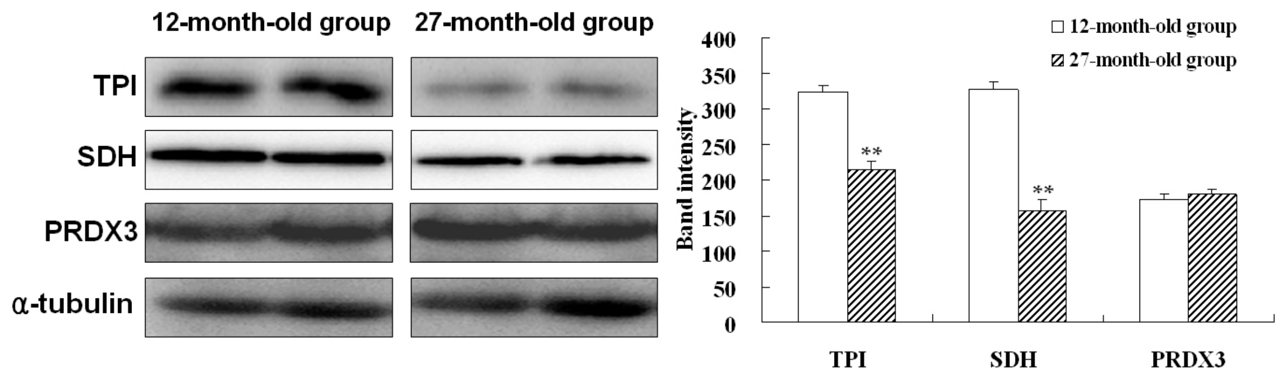

Fig. 3. Expression patterns of triosephosphate isomerase (TPI), succinate dehydrogenase (SDH) and thioredoxin-dependent peroxide reductase (PRDX3) in 12 and 27-month-old groups. **Significant differences ($P < 0.01$) between 12 and 27-month-old groups were determined using the t-test module on SAS.

loss of myofibril proteins in loin muscle during the fattening stage may be due to an increase in adipogenesis in muscle tissue.

The ZNF configuration is a common DNA-binding motif of transcription regulatory proteins in the cell nucleus (19). The ZNF domain mainly functions in the promoter regions of cell differentiation and developmentally related genes, resulting in the specific activation and repression of gene expression (19). Pi *et al*. (20) reported that ZNF323 and the ZNF-containing SCAN Box domain were more weakly expressed in muscle than in other tissues. A member of the SCAN-domain family was previously demonstrated to be preferentially bound by PPARγ as an adipogenic cofactor (21), and the expression of the ZNF protein was reported to increase during adipocyte differentiation (22, 23). These results suggest that the ZNF protein may be positively associated with adipogenesis at the cellular level. At present, the biological function of ZNF323 in muscle is unclear, but the ZNF323 protein may be an essential factor regulating adipogenic-related gene expression during the late fattening stage of animals.

In this study, we conclude that actin, MLC1, TPI, SDH, and ZNF323 were differentially expressed proteins that may be associated with fat deposition in Hanwoo (Korean cattle) steer. Particularly, TPI and SDH may prove to be valuable as markers of intramuscular fat-related protein. Future studies will confirm the expression of these proteins in m. *longissimus dorsi* muscle of animals with high and low intramuscular fat.

MATERIALS AND METHODS

Animals and sample preparation

Samples of *m. longissimus dorsi* muscle from 12- and 27-month-old Hanwoo (n = 6 animals of each group) steers were obtained at the junction between the 11th and 12th lumbar vertebrae within 40 min after slaughter. The selected tissues were placed in liquid nitrogen, ground to a fine powder using a mortar, and stored at −80°C for mitochondrial proteome and crude fat content analysis. Crude fat content in the longissimus dorsi muscle was analyzed using the methods of Folch *et al*. (24).

The muscle samples (1.0 g) were homogenized in 10 ml of medium A [120 mM KCl, 20 mM 4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid (HEPES; pH 7.4), 2 mM $MgCl_2$, and 1 mM ethylene glycol tetraacetic acid (EGTA)], on ice using a Dounce tissue grinder (Sigma-Aldrich, St. Louis, MO, USA). The homogenate was centrifuged at 600 × g for 10 min, and the supernatant decanted into a fresh tube. The pellet was resuspended in 5 ml of medium A and recentrifuged. The second supernatant was combined with the first and the mixture was centrifuged at 17,000 × g for 10 min. The pellet from the supernatant mixture was again resuspended in 5 ml of medium A and centrifuged at 7,000 × g for 10 min. This pellet was resuspended in medium B [300 mM sucrose, 2 mM HEPES (pH 7.4), and 0.1 mM EGTA] and centrifuged at 3,500 × g for 10 min. The resulting pellet containing the mitochondrial proteins was suspended in 0.3 ml of lysis buffer [7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 40 mM Tris, 1% dithiothreitol (DTT), and a protease inhibitor cocktail; Roche Diagnostics GmbH, Mannheim, Germany]. The protein content was determined using a protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin (BSA) as the standard.

Two-dimensional electrophoresis (2-DE) and image analysis

Approximately 200 μg of individual protein sample was applied onto 17 cm, immobilized pH gradient (IPG) strips (3-10 nonlinear; Bio-Rad). The IPG strips were rehydrated overnight in a solution containing 7 M urea, 2 M thiourea, 2% CHAPS, bromophenol blue (a few grains), and 2 mM tributylphosphine. After rehydration, isoelectric focusing (IEF) was performed for 42,000 Vh with a PROTEAN IEF Cell unit (Bio-Rad) at 100, 250, 500, and 1,000 V for 1 h, according to the manufacturer's instructions, and then the voltage was gradually increased to 8,000 V. The current limit was adjusted to 50 μA/strip, and the run was carried out at 20°C.

After IEF, the IPG strips were incubated for 20 min in 10 ml of equilibration solution that consisted of 50 mM Tris-Cl (pH 8.8), 6 M urea, 2% SDS, 20% glycerol, 0.01% bromophenol blue, and 5 mM tributylphosphine. The incubated IPG strips were transferred onto SDS-PAGE gels (12% T and 2.67% C) using 5 mA/gel for 1 h followed by 15 mA/gel until the dye front reached the bottom of the gel. The gels were fixed for 1 h in a solution of 40% ethanol and 10% acetic acid and then stained using the Blum silver-stain protocol (25). Stained gels were matched and analyzed with PDQuest software (Bio-Rad). The 2-DE analysis was repeated three times for each sample. For comparative analysis, the relative densities of individual spots were analyzed and compared between the two study groups. Spot intensities were normalized to the total intensity. First, the spots that were present in at least 2 gels (n = 3 gels of each sample) were considered as the candidate spots. Then the differentially expressed spots with more than a twofold change in intensity between the 12- and 27-month-old groups were used for the comparison. The averaged spot densities were submitted to one-way analysis of variance (ANOVA) using SAS software (26). Densities were considered significant at the $P < 0.05$ level.

Protein identification

The differentially expressed spots were excised from the silver-stained gels and destained with 30 mM potassium ferricyanide and 100 mM sodium thiosulfate (1 : 1). The destained samples were in-gel reduced and alkylated with DTT and iodoacetamide in an ammonium bicarbonate solution, digested with 20 μl of trypsin solution (7 ng/μl trypsin in 50 mM ammonium bicarbonate), and incubated for 16 h at 37°C. After enzymatic digestion, the sample was extracted once with 50 μl of 50 mM ammonium bicarbonate and twice with 50 μl of 0.1% formic acid in 50% acetonitrile. The extracted solutions were dried in a vacuum centrifuge. Samples were rehydrated in 30 μl of 0.5% trifluoroacetic acid (TFA), desalted using Poros R2 resin (Perseptive Biosystems, Framingham, MA, USA), and eluted with 2 μl of 60% methanol containing 5% formic acid prior to mass spectrometric (MS) analysis.

The mass of each sample was obtained with the 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA) in the positive-ion reflector mode. The tandem MS (MS/MS) analysis was performed with the five most abundant ions and the proteins were identified by searching SWISS-PROT and NCBI databases using the Mascot peptide mass fingerprinting and ion search programs (Matrix Science, London, UK).

Western blotting

For western blotting, 40 μg of sample proteins (n = 6 for each group) were separated on SDS-PAGE according to Laemmli (27) and gels were transferred to PVDF membranes (Millipore) in ice-cold transfer buffer (25 mM Tris-Cl, pH 8.3, 1.4% glycine, 20% methanol) at 250 mA for 90 min. Membranes were treated with blocking buffer containing 5% non-fat milk (Becton, Dickinson and Company, MD, USA) in TBS/T (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) and incubated overnight at 4°C. Primary goat mouse-SDH (sc-59687), goat anti-TPI (sc-22031) and goat-anti-PRX (sc-23969) antibodies, all from Santa Cruz Biotechnology, Inc. (Santa

Cruz, CA, USA), were used at 1 : 200 dilutions in TBS/T with 5% non-fat milk. Following 2 h of incubation with primary antibodies, membranes were washed three times for 10 min each with 10 ml of TBS/T. Horseradish peroxidase-labeled (HRP) anti-goat secondary antibody was diluted 1 : 5,000 in TBS/T with 5% non-fat milk and incubated with the membranes for 1 h. After three 10-min washes, membranes were visualized using a chemiluminescent HRP substrate (Millipore) and a VersaDoc imaging system (Bio-Rad). The band densities were calculated by Quantity One software (Bio-Rad) and normalized by density of α-tubulin (sc-12462, Santa Cruz Biotechnology, Inc.). All experiments were repeated in triplicate.

Statistical analyses

The student t-test was performed for the identification of differentially expressed proteins between 12 and 27-month-old groups. The correlation between differentially expressed protein and fat content was analyzed by a Pearson correlation. All statistical analysis in this study was performed using the SAS statistical package (26).

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